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Specific Enzyme-Multiplied Immunoassay and Fluorescence Polarization Immunoassay for Cyclosporin Compared with Cyclotrac [^{125}I]Radioimmunoassay

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Summary: The analysis of cyclosporin-A (CsA) has proved a valuable adjunct to clinical care of patients who have received organ grafts. The measurement of CsA in whole blood by specific methods has recently taken a new direction with the introduction of a range of rapid methods, including a homogeneous enzyme immunoassay technique (EMIT) and a monoclonal fluorescence polarization immunoassay (FPIA). The present paper compares these two methods with the established Cyclotrac specific [^{125}I]RIA (radioimmunoassay) using both commercial CsA-spiked control material as well as a group of 60 patient specimens (predominantly renal transplants). While each of the new methods showed acceptable precision and accuracy with the commercial quality control material, significant differences were demonstrated with patient specimens, such that FPIA was 12.5% greater than [^{125}I]RIA ($p < 0.0001$), which was in turn 5.9% greater than EMIT ($p = 0.007$). These data suggested that the FPIA may have residual CsA-metabolite interference and that the EMIT method was the most "specific" for parent CsA of the three tested, potentially therefore more comparable to high-performance liquid chromatography (HPLC). Key Words: Cyclosporin—Immunoassay—Enzyme-multiplied immunoassay—Fluorescence polarization immunoassay—Radioimmunoassay—Therapeutic drug monitoring.

The analysis of the immunosuppressant drug, cyclosporin-A (CsA), has received intensive scrutiny in recent years, particularly from the perspective of the therapeutic drug monitoring (TDM) laboratory where the aim is to report a CsA concentration that will positively assist in clinical therapeutic decisions in patients with a grafted organ(s). The analytical debate has been focused on a range of issues, including (a) the matrix to be assayed (i.e., plasma, serum, or whole blood), (b) the anticoagulant to be used, (c) whether to measure "total" CsA-related substances (i.e., parent drug plus CsA-metabolites) or parent CsA alone, (d) whether a single trough sample is representative of concentrations over the preceding dosing interval, (e) whether the analytical result has predictable relationship to the pharmacological response, i.e., whether there exists a "therapeutic range" (1) which can exclude, or at least minimize, the incidence of CsA-related side-effects, (f) whether such a range needs to be modulated for different transplanted organs (or even other applications of this drug) or for duration of the graft (i.e., whether a lower circulating CsA concentration can maintain adequate immunosuppression for the

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grafted organ after 3 to 6 months). The reader is referred to the recent Canadian Consensus Meeting on Cyclosporin Monitoring which pursues these matters in detail (2).

When CsA became widely used clinically in the early 1980s, the analytical choice was essentially between the nonspecific polyclonal radioimmunoassay ([^{3}H]RIA) marketed by Sandoz (3), which measured parent CsA with significant cross-reactivity with a range of CsA-metabolites (this method being later withdrawn), and specific high-performance liquid chromatographic (HPLC) methods, which quantified parent CsA independently from CsA-metabolites (4). The latter proved challenging to most laboratories as exemplified by the plethera of HPLC methods reported [e.g., (5,6)]. The polyclonal nonspecific [^{3}H]RIA was replaced with monoclonal antibody (7) [^{3}H]RIAs of Sandoz and [^{125}I]RIAs of Incstar Corporation (8) each of which offered a choice of specific or nonspecific antibodies for measuring parent CsA with, or independently from CsA-metabolites (9–11). There was, however, some concern that the [^{125}I]RIA had a positive bias over HPLC (12), but this was contested by the manufacturer (Bucholz, personal communication, 1989) and others (6). The magnitude of this proposed bias was small (10–20%) and unlikely to be of major clinical consequence, particularly when compared to the nonspecific assays, such as the polyclonal nonspecific fluorescence polarization immunoassay (FPIA) run on the TDx analyzer that was introduced at about the same time by Abbott Diagnostics (estimated to be 300–400% in this laboratory in renal transplant patients and due to CsA-metabolite cross-reactivity). This nonspecific FPIA method was marketed as offering a rapid turnaround time compared with either HPLC or the RIAs (13,14). This proved attractive to many TDM laboratories already running such analyzers.

There was therefore a dilemma facing the TDM laboratory, as well as the clinicians responsible for transplanted patients. The potential existed for the transplanted patient's blood specimens to be assayed by a spectrum of laboratories, e.g., if the patient were relocated to a major medical center for the transplantation and subsequently returned home (possibly to a regional center) for long-term care. These laboratories could well be utilizing alternative methods and therefore generating "CsA concentrations" varying by 4-fold where such data should be comparable if one is to avoid confusion in clinical management. Obviously the clinical interpretation and consequences of such results could be dramatic. As a guide to TDM laboratories, the National Academy of Clinical Biochemistry, together with the American Association of Clinical Chemistry formed a task force to consider the CsA analytical issues and their report was a landmark in CsA determination (15). More recently the Canadian Consensus Meeting (2) (alluded to above) has supported and extended these recommendations. These groups, and others (16) proposed, among other things, that laboratories should measure parent CsA in whole blood (with EDTA anticoagulant). If the TDM laboratory were to adopt these recommendations, then the choice would be between the more labor-intensive RIAs or HPLC, neither of which offered a rapid turnaround time, or "stat" assay capability.

With this wide range of methods, matrices, etc., for CsA measurement, the need for ongoing quality assurance testing is paramount (17,18). These authors allude to the strong trend of laboratories toward specific methods for CsA determination and to the use of whole blood rather than plasma for TDM of CsA.

This situation is now being remedied by the introduction of a choice of rapid, specific, nonradiochemical methods by Syva, Abbott, and Du Pont to be run on the Cobas MIRA (Roche Diagnostics), the TDx analyzer (19,20) and ACA analyzer (21), respectively, as well as the fluorogenic enzyme-linked immunosorbent assay (ELISA) dry film method on the OPUS immunoassay system. The first two are considered in this communication (the third manufacturer declined an offer to be included in this study). These two monoclonal "specific" methods are compared with each other and with the established specific [^{125}I]RIA of Incstar Corporation (Cyclotrac SP). Precision and accuracy are considered using independent commercially prepared control material and a correlation study using 60 patient specimens (predominantly renal transplants) assayed by each of the three methods.

MATERIALS AND METHODS

Three commercial methods for the determination of CsA were included in the study. The two new nonisotopic methods considered were EMIT (Syva, Palo Alto, U.S.A.) run on a Cobas MIRA analyzer (Roche Diagnostics Division, Nutley, NJ, U.S.A.)

and FPIA run on a TDx analyzer (Abbott Diagnostics Division, North Chicago, IL, U.S.A.), were compared with the established ^{125}I-RIA (Cyclotrac SP, Incstar Corporation, Stillwater, MN, U.S.A.). Each of the methods were performed precisely as prescribed by the respective manufacturer in the kit package literature.

Homogeneous Enzyme Immunoassay

The EMIT assay requires a 100 μl aliquot (using a positive displacement pipette) of whole blood (calibration standard, control or patient specimen) to be diluted into 200 μl of methanol, this dilution is vigorously mixed, allowed to stand for 2 min and microcentrifuged at >8,000 *g* for 2 min. The purpose of this pretreatment step is to lyse the red blood cells, solubilize the CsA, and precipitate the protein. The supernatant (100 μl) is then placed into the appropriate sample tube together with 200 μl of diluent (Tris buffer, surfactant, and preservatives; concentration and pH not supplied) for analysis on the MIRA analyzer. Quantitation was based on calibration standards (batch number 6R119-C1) and reagents (batch numbers 6R019-C1G and D1G) provided by the same manufacturer assayed in parallel in each assay run. The six calibration standards provided in this kit are, 0, 50, 100, 200, 350, 500 μg/L with sodium azide added.

Fluorescence Polarization Immunoassay

The FPIA method (Abbott Diagnostics, reagent lot number 50006SV and calibration standards lot number 50148SV) requires a 150 μl whole blood aliquot (calibration standard, control or patient specimen) to be mixed with 50 μl of "solubilization" reagent (aqueous surfactant with 0.1% sodium azide) and 300 μl of precipitation reagent (zinc sulfate in methanol and ethylene glycol; concentrations not supplied). Following a vigorous mixing, this solution is centrifuged at 9,500 *g* for 5 min. The supernatant is decanted into sample cups, loaded in the TDx analyzer and thereafter automatically quantified. The reagents provided in this kit include CsA antibody (<25% mouse monoclonal in a buffer containing stabilizer with 0.1% sodium azide), and a <0.01% CsA monoclonal whole blood fluorescein tracer solution in buffer containing surfactant and protein stabilizer with 0.1% sodium azide. Six calibration standards are provided in this kit, 0, 100, 250, 500, 1000, 1500 μg/L with sodium azide (0.1%) (22).

Patient Specimens

Sixty whole blood specimens, used as the basis for correlation, were largely from renal transplant recipients (97%); the remainder were bone marrow transplants. These specimens were selected on the basis of the [^{125}I]RIA result so as to include 20 in each of the three ranges, <200, 200 to 400, and >400 μg/L, and hence they spanned the concentration range typically experienced in the TDM laboratory. Specimens were stored below −20°C throughout the study period. Those which exceeded the top calibration standard for the EMIT method (i.e., 500 μg/L) were diluted (1 part in 4), using CsA-free whole blood, for assay.

Quality Control, Precision, and Accuracy

All assays were controlled by running in parallel independent commercial trilevel quality control material (Lyphochek Whole Blood Control, BioRad, lot number 43801, 43802, 43803 for FPIA, and lot number 47001, 47002, 47003 for EMIT assays) in each run of each of the three methods. These same materials were also used to provide an index of both precision and accuracy for the two new methods. Specifically, each of the new methods was subjected to within- and between-run precision and accuracy studies. The within-run reproducibility was assessed by assaying 20 replicates of each of the three control blood samples in a single run. The between-run reproducibility was tested by assaying each of the three control samples in duplicate both in the morning and afternoon of 20 consecutive working days.

Dilution Study

To consider the detection limit for each of the test methods, the 200 and 250 μg/L calibration standards for the EMIT and FPIA methods, respectively, were serially diluted down to approximately 10 μg/L using CsA-free whole blood, and each of these samples assayed in duplicate as "unknowns" through the established procedure.

Statistical Considerations

Each of the test methods were compared to the established RIA method, as well as to each other, using Student's paired *t*-test. The means were used to assign the bias of the particular method as com-

pared with RIA. Linear regression analysis was used for the correlation studies to consider the coefficient of determination (r^2) for each relationship. The 5% level was assigned as the index of significance. In addition, the ratio of each test method to the RIA was plotted against the RIA result to further illustrate the deviation from unity.

RESULTS

Precision and Accuracy

The results of precision and accuracy testing using the commercial trilevel quality control material are presented in Table 1. This shows that within-run (n = 20) coefficients of variation expressed as a percentage (CV%) data for the EMIT and FPIA methods were 7.2, 4.5, and 6.6%; and 4.3, 2.7, and 1.9%, respectively, for concentrations spanning each calibration range (discussed below). The comparative between-run (n = 40) values were 13.2, 7.0, and 10.3%; and 10.0, 6.1, and 6.0%, respectively. Accuracy was determined by the deviation from "target" concentrations for each of the three quality control specimens when assayed by each method. These data are also presented in Table 1 and show that mean deviations for the EMIT method from the target concentration for the intra- and interassay data were −11.8, +6.2, and +3.6%, and +5.5, +1.5, and −4.7%, respectively. The "target" concentrations for HPLC analysis provided by the manufacturer of this control material were adopted as the basis for comparison for the FPIA as there was no such target available for FPIA. The FPIA data showed deviations of −6.5, −4.8, and −3.3%, and −7.3, +3.4, and +6.7% for the intra- and interassay trilevel controls, respectively. These data suggested that both methods achieved the "target" concentrations consistently. It should be noted that different batches of this commercial quality control material were used for the EMIT and FPIA analyses, in each case to test the calibration curve of the particular method which spanned quite different ranges (i.e., 0 to 500 μg/L for EMIT, and 0 to 1500 μg/L for FPIA).

Correlation with [^{125}I]RIA

The correlation data using the 60 transplant patient specimens (predominantly renal) are presented in Figs. 1–3 and demonstrate highly significant correlations between the methods; however, there were significant differences (using paired Student's *t*-testing) between the three methods as follows. Compared with [^{125}I]RIA, the EMIT assay had a coefficient of determination (r^2) of 0.98 and a mean bias of −5.9% (using mean concentrations of assayed specimens, $t_{59} = 2.79$, $p = 0.007$). Similarly, the FPIA had a coefficient of determination (r^2) of 0.98 and a mean bias of +12.5% ($t_{59} = 7.24$, $p < 0.0001$) compared with [^{125}I]RIA. Hence the relationship between the two test nonradiochemical methods was a 18.4% bias ($t_{59} = 7.94$, $p < 0.0001$)

TABLE 1. *Precision and accuracy data for the specific EMIT and FPIA CsA assays*[a]

	Within-run					Between-run				
QC[b]	Mean	SD	Range	CV%	Bias%	Mean	SD	Range	CV%	Bias%
EMIT assays:										
Level 1 (78 μg/L)	68.8	4.95	62–78	7.2	−11.8	82.3	10.9	60–110	13.3	+5.5
Level 2 (195 μg/L)	207	9.26	189–229	4.5	+6.2	198	14.7	156–228	7.4	+1.5
Level 3 (360 μg/L)	373	24.7	340–431	6.6	+3.6	343	35.0	220–406	10.2	−4.7
FPIA assays:										
Level 1 (93 μg/L)	87.5	3.79	79–93	4.3	−6.5	86.2	8.63	70–107	10.0	−7.3
Level 2 (207 μg/L)	197	5.31	187–206	2.7	−4.8	214	13.0	197–244	6.1	+3.4
Level 3 (652 μg/L)	637	12.4	620–664	1.9	−3.3	696	41.5	627–789	6.0	+6.7

[a] The testing was undertaken using commercial trilevel control material (note that the batch number was different for the two methods; see text).
[b] The commercial control material used was from different batches for the EMIT and FPIA assays, hence the apparent discrepancy between the two methods in target and measured concentrations, particularly in level 3.

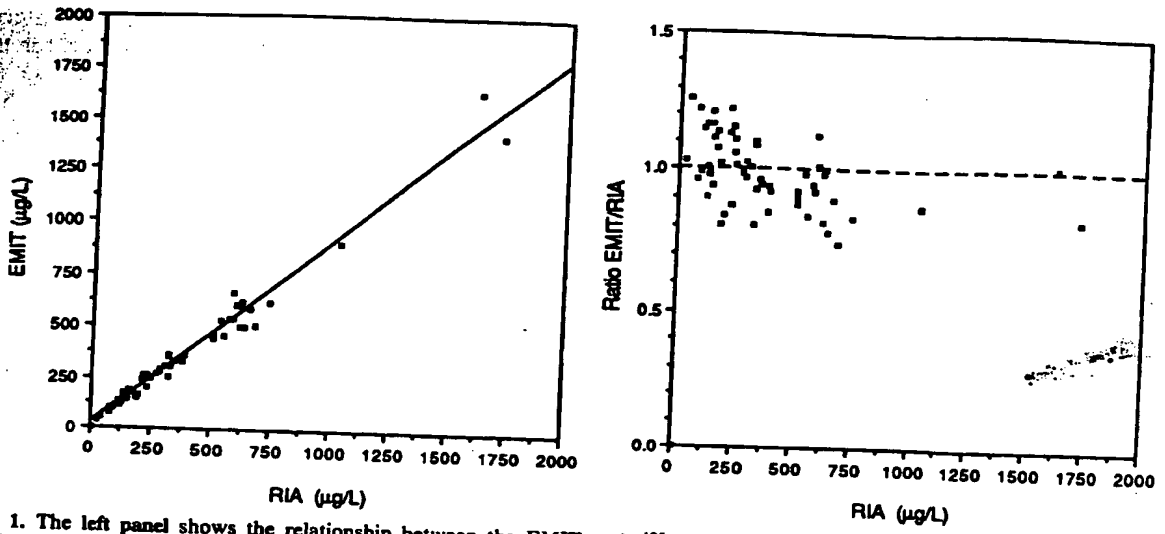

FIG. 1. The left panel shows the relationship between the EMIT and [^{125}I]RIA methods as assessed using 60 patient specimens (predominantly renal transplants). The regression line has the equation, $y = 22.5 + 0.88x$ with a coefficient of determination (r^2) of 0.975. The right panel shows the ratio of the EMIT to the RIA value for each specimen plotted against the RIA value. The dashed line is the identity line (ratio of unity).

of the FPIA over the EMIT assay. To account for the potential contribution of the few very high concentration data points (i.e., >800 µg/L), the correlations were repeated with these points excluded, in which case the coefficients of determination were only slightly reduced to 0.96 and 0.95 for the EMIT and FPIA comparisons with RIA, respectively. The ratio plots (EMIT/RIA and FPIA/RIA) also included in Figs. 1 and 2 showed the scatter of values around the ideal (i.e., ratio of unity) value. These data suggested, in the case of the EMIT assay, possibly a greater distribution of "high" EMIT values within the range of the calibration curve (<500 µg/L) than at higher concentrations. This feature was

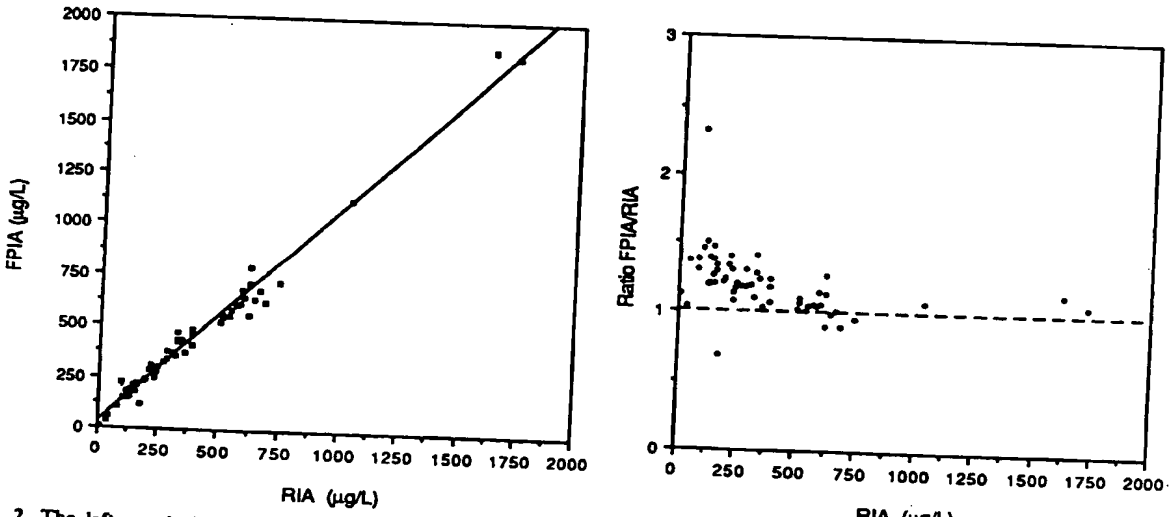

FIG. 2. The left panel shows the relationship between the FPIA and [^{125}I]RIA methods as assessed using 60 patient specimens (predominantly renal transplants). The regression line has the equation, $y = 29.8 + 1.05x$ with a coefficient of determination (r^2) of 0.981. The right panel shows the ratio of the FPIA to the RIA value for each specimen plotted against the RIA value. The dashed line is the identity line (ratio of unity).

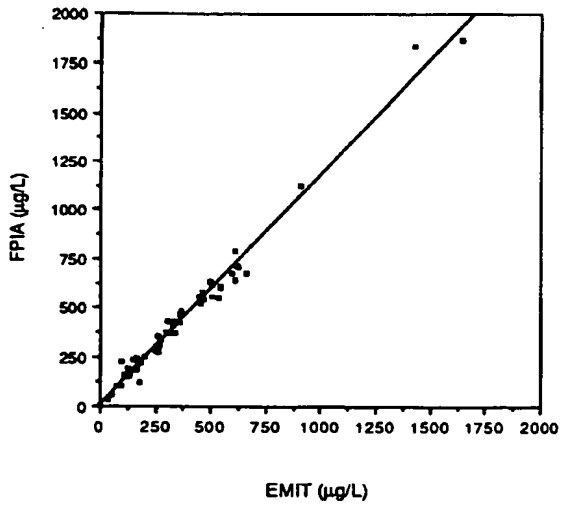

FIG. 3. The plot shows the relationship between the EMIT and FPIA methods as assessed using 60 patient specimens (predominantly renal transplants). The regression line has the equation, $y = 7.82 + 1.17x$ with a coefficient of determination (r^2) of 0.984.

more pronounced with the FPIA where almost all of the data below 500 µg/L were well above the unity line.

Dilution Study

While not a definitive means of estimating the limit of detection for either method, the serial dilution of a calibration standard, presented in Table 2, suggests that the EMIT method accuracy is still relatively good at 12.5 µg/L (the low calibration standard being 50 µg/L), and the FPIA deteriorated at approximately 100 µg/L (the low calibration standard being 100 µg/L). The sensitivity limits proposed by each manufacturer were 25 µg/L and 12–31 µg/L for FPIA and EMIT methods, respectively.

DISCUSSION

The data presented suggests that both EMIT and FPIA CsA methods had adequate precision and accuracy for the purposes of routine therapeutic drug monitoring. The precision data obtained for the FPIA was consistently better than the EMIT method in this study. This EMIT CV data is compared with that reported (23) where with-in run CVs of 5.0 to 7.1%, and between-run CVs of 4.9 to 7.4% were observed. While the within-run EMIT CV data in the present study compare well with these data (23) from the manufacturer's laboratories, their between-run CV data is clearly superior to that in the present study. The manufacturer has acknowledged that users may experience between-run CV data of approximately 10% at the low end of the calibration curve (personal communication, 1991).

The selection of calibration standards for the FPIA method which has a low calibration standard of 100 µg/L, could be considered not low enough by many laboratories, and three of the five "nonzero" calibration standards were greater than the "therapeutic range" used in this laboratory (80 to 250 µg/L) and elsewhere (16). Hence these calibration standards for the FPIA method would not adequately cover either the low end or therapeutic concentrations of the anticipated patient specimens, as reporting a result of <100 µg/L (i.e., below the defined calibration limit) would not adequately discriminate those patients who were (a) adequately maintained at the low end of this therapeutic range or (b) "sub-therapeutic," or (c) clearly noncompliant. The selected range appears to be more closely related to this manufacturer's previous nonspecific polyclonal FPIA where a 3- to 4-fold greater assay result would have been anticipated.

Discrepancies which appeared during the correlation studies using 60 patient specimens were of considerable interest. The obvious basic difference between the patient specimens used for the correlation, and the quality control material used for precision and accuracy studies, being the presence of CsA-metabolites in the patient samples. It is proposed that the statistically significant ($p < 0.001$)

TABLE 2. *Results of dilution studies using a calibration standard for each of the test methods*

	FPIA			EMIT		
Dilution	Expected	Observed	Diff.(%)	Expected	Observed	Diff.(%)
a. undiluted	250	—	—	200	195	−2.5
b. 1:1 of a	125	116	−7.2	100	108	+7.5
c. 1:1 of b	62.5	51	−18.4	50	53.8	+7.6
d. 1:1 of c	31.3	17	−45.7	25	19.8	−20.8
e. 1:1 of d	15.6	10	−35.9	12.5	12.7	+1.6
f. 1:1 of e	7.8	<1		—	—	—

deviation of the FPIA from both the EMIT and [^{125}I]RIA methods in patient specimens may relate to residual CsA-metabolite cross-reactivity by the FPIA, as no such deviation was apparent in the CsA-metabolite-free control material where results compared favorably with HPLC target values. Such interference has also recently been demonstrated in the international quality assurance program based at St. George's Hospital, London (UKQAP) where specimens containing CsA metabolites M-1 and M-17 (also referred to as AM9 and AM1, respectively), without parent CsA, were distributed for testing. The monoclonal FPIA results from 26 laboratories showed mean data ranging from 2.1- to 6.3-fold greater than HPLC, [^{125}I]RIA, or EMIT, strongly suggesting a residual level of CsA-metabolite interference. Interestingly, the literature provided to this laboratory by Abbott titled "Introducing TDx Cyclosporine Monoclonal Whole Blood" (booklet #85-4821/R1) makes no reference to the method as being "specific" for parent CsA, or as being free from cross-reactivity with CsA-metabolites (remembering that both Sandoz and Incstar market monoclonal nonspecific RIAs for CsA). Neither does this literature indicate the anticipated CsA-metabolite cross-reactivities. This would appear to be a significant oversight given the debate which has taken place over recent years on this matter. Alternatively, it could be postulated that this manufacturer accepts that its method has significant residual CsA-metabolite cross-reactivity, albeit at a much lower level than its previous polyclonal nonspecific method, and possibly assuming that this apparent residual cross-reactivity was not of major clinical consequence for many patients. The slightly lower result with EMIT compared with [^{125}I]RIA in the present study could be interpreted as consistent with the proposed positive bias of this [^{125}I]RIA over HPLC (12). The implication therefore being that EMIT would compare favorably with HPLC.

In summary, the data presented in this comparative study demonstrate that both the FPIA and EMIT methods have successfully brought a rapid CsA assay to the therapeutic drug monitoring laboratory each with relative advantages and disadvantages as discussed in detail above. In this country, it would appear that kit costs are likely to be similar, but each somewhat dearer than the [^{125}I]RIA. Some of this initial capital outlay difference would, however, be absorbed into a reduced number of calibration standard assays with the nonradiochemical methods which offer minimum calibration curve stabilities of a few weeks, as compared with routine calibration in each run by RIA. Patient benefits could result from a faster turnaround time in the ward and stat-assay capabilities in the acute care situation. The time commitment of laboratory staff with either of the new CsA methods would be considerably reduced compared with either RIA or HPLC. On balance, it is the opinion of these authors that for laboratories with access to a Cobas MIRA analyzer, the EMIT method has considerable advantages over the monoclonal FPIA and [^{125}I]RIA, and also HPLC for routine TDM purposes.

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